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Review

Protein engineering of cytochromes *P*-450

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Abstract

The cytochromes *P*-450 are an immensely important superfamily of heme-containing enzymes. They catalyze the monooxygenation of an enormous range of substrates. In bacteria, cytochromes *P*-450 are known to catalyze the hydroxylation of environmentally significant substrates such as camphor, phenolic compounds and many herbicides. In eukaryotes, these enzymes perform key roles in the synthesis and interconversion of steroids, while in mammals hepatic cytochromes *P*-450 are vital for the detoxification of many drugs. As such, the cytochromes *P*-450 are of considerable interest in medicine and biotechnology and are obvious targets for protein engineering. The purpose of this article is to illustrate the ways in which protein engineering has been used to investigate and modify the properties of cytochromes *P*-450. Illustrative examples include: the manipulation of substrate selectivity and regiospecificity, the alteration of membrane binding properties, and probing the route of electron transfer.

Keywords: *P*-450; Protein engineering; Substrate specificity; Electron transfer; Chimeragenesis; Genetic fusion

1. Introduction

Protein engineering is generally understood to mean the use of site-directed or random mutagenesis to alter the properties of a protein or enzyme. This often involves the modulation of substrate specificity or selectivity of an enzyme. In this review we have extended the definition to encompass other important aspects of structure/function investigations, particularly the construction of chimeras and genetic fusions to investigate substrate specificity and electron transfer, and the modification of the N-terminal membrane anchor of eukaryotic *P*-450s to facilitate overexpression and for the investigation of protein targeting. In the course of the review we will use specific examples to address these topics and highlight the most significant breakthroughs made in recent years. The variation of protein engineering experiments applied to the cytochromes *P*-450 is a reflection of the medical and technological importance of these enzymes. It is useful, therefore, to briefly consider the properties of the cytochromes *P*-450 and some of the major advances in our understanding of their biological roles and catalytic mechanisms.

The cytochromes *P*-450 are a family of *b*-type heme containing proteins found in virtually every organism [1]. They catalyze the monooxygenation

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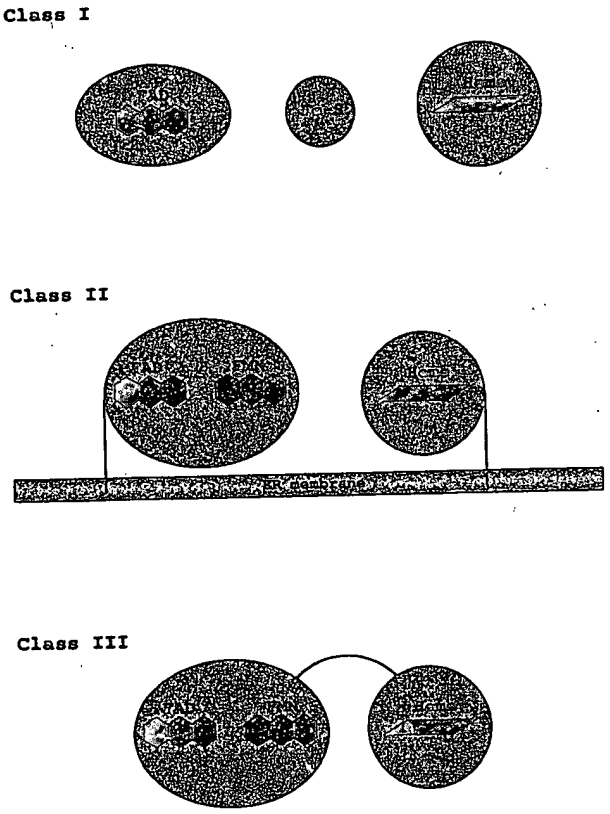


Fig. 1. Schematic representation of the different classes of cytochrome *P*-450 systems. Class I systems are composed of an FAD containing flavodoxin reductase, an iron-sulfur cluster containing flavodoxin and the *P*-450. The *P*-450 in a class II system is partnered by a diflavin reductase, and in a class III system, the diflavin reductase is fused to the *P*-450 in a single polypeptide.

of a diverse array of aromatic and aliphatic compounds in numerous biosynthetic and metabolic pathways. In mammals, they are central to the processes of drug metabolism and steroid hormone synthesis. In plants they play important roles in herbicide resistance and biosynthetic pathways (e.g. morphine biosynthesis), they are involved in antibiotic synthesis in fungi, plant toxin defence in insects, and in the metabolic and synthetic pathways of bacteria. The most common reaction catalyzed is hydroxylation (see below) but forms that catalyze epoxidation, dealkylation and sulfoxidation are known.

$$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O$$

The *P*-450 reaction requires two electrons (provided by redox partner proteins), dioxygen and two protons. It proceeds via an activated oxygen species that effects controlled insertion of an oxygen atom into the substrate. Most bacterial and all of the eukaryotic mitochondrial *P*-450 systems are three component protein systems (class I): the redox partners consist of an NADH-dependent FAD containing ferredoxin reductase and a ferredoxin. Eukaryotic microsomal *P*-450 systems (those associated with the endoplasmic reticulum) are two component systems (class II): the redox partner is an NADPH-dependent diflavin (FAD and FMN containing) reductase. A third class (class III), of which the representative system is the bacterial *P*-450 BM3, is a one component system similar to class II but where the two components are fused in a single polypeptide. A schematic depiction of these system types [2] is illustrated in Fig. 1. In the *P*-450 itself, in all of these system classes, the heme iron is proximally ligated by a cysteine-thiolate, and in the resting state, the ferric iron is distally ligated by water. The reaction cycle is

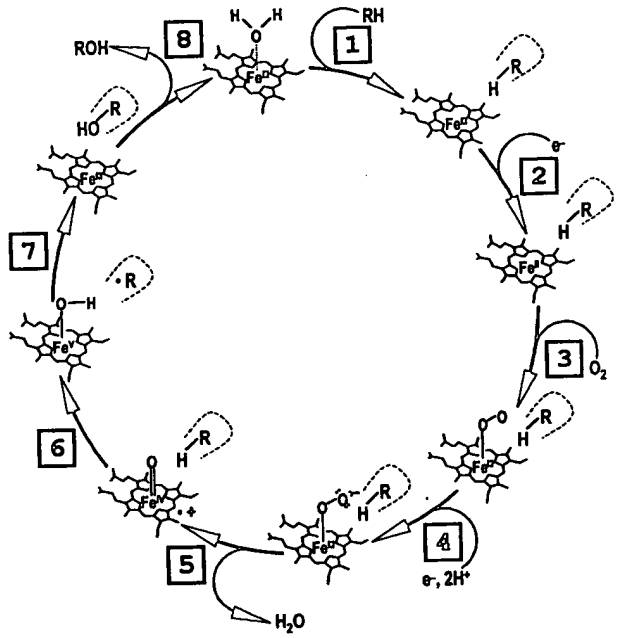


Fig. 2. The catalytic cycle of cytochrome *P*-450. The cycle is initiated by substrate (RH) binding to the ferric *P*-450 (1), permitting the first electron transfer (2) with consequent iron reduction and binding of oxygen (3). Second electron transfer (4) results in formation of the iron-peroxo species (4) and subsequently the oxyferryl intermediate (5). This reacts with bound substrate (6) and (7) to yield hydroxylated product that diffuses from the active site to complete the cycle (8).

represented in Fig. 2. Mechanistic information on this cycle has come largely from studies on the *P*-450cam system, and is generally assumed to hold for all *P*-450s [3]. The cycle is initiated by substrate binding close to the heme, which usually induces a change in the spin state of the iron from low to high spin. The iron is reduced to the ferrous form by the transfer of one electron from a redox partner and oxygen binds rapidly. The second electron transfer to the heme results in the cleavage of dioxygen and the formation of a transient oxy-ferryl intermediate (similar to compound I seen in peroxidases) and the release of water. Ultimately, the reactive oxyferryl species attacks the substrate, resulting in the mono-oxygenation of the compound.

The number of *P*-450 genes now recognized is enormous and genome sequencing projects continue to add to this on a day-to-day basis. The sequences have been organized into what is now termed the *P*-450 gene 'superfamily' [4] (see Kirill Degtyarenko's Directory of *P*-450 systems at http://www.icgeb.trieste.it/~p450srv/and David Nelson's *P*-450 home page at http://drnelson.utmem.edu/CytochromeP450.html), allowing a greater understanding of the evolution of the *P*-450s from primordial forms. The presence of a *P*-450 in the archaeon *Sulfolobus solfataricus* indicates that they are ancient enzymes [5]. It is possible that they arose as a means for breaking down toxic oxygen when it first became abundant on earth, and an early physiological role may have been the modification of lipids for the cell membranes of microbes.

The presence of *P*-450s in organisms as diverse as bacteria and man is a reflection of the importance of the oxygen cleavage reaction that they catalyze. Through evolution, nature has adapted this reaction to suit the requirements of numerous different synthetic and degradative pathways. *P*-450s are adapted to act on molecules as small as nitric oxide (*P*-450nor from the fungus *Fusarium oxysporum*) [6] and as large as cholesterol and polycyclic aromatic hydrocarbons (e.g. mammalian *P*-450s scc and 1A1) [7,8]. This substrate diversity demonstrates the potential for strategic engineering to provide a route for the generation of *P*-450 enzymes tailored to a chosen molecule, enabling the regio- and stereoselective oxygenation of new substrates. The reactivity of oxygen makes selective oxygenation difficult to achieve by conventional chemical means. This is one area of great interest in the field of *P*-450 engineering.

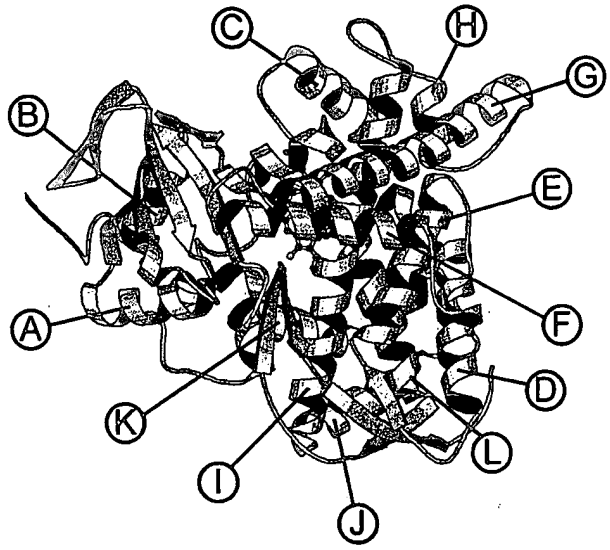


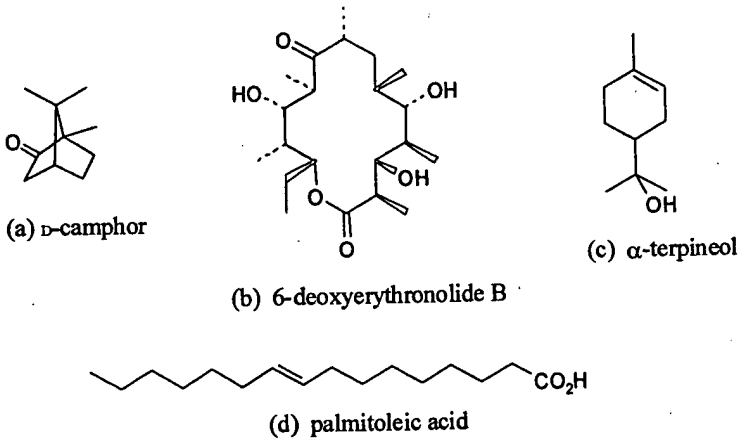
Fig. 3. Schematic representation of the heme domain of flavocytochrome *P*-450 BM3, showing the characteristic *P*-450 fold, produced using MOLSCRIPT [153]. The heme cofactor is visible as a ball-and-stick structure and helices A–L are labeled.

1.1. The structural basis for cytochrome P-450 engineering

Rational re-design or re-engineering of an enzyme requires a firm structural basis. At present there are eight atomic structures for different cytochromes *P*-450. Only five of these are freely available at present, although two others (mammalian *P*-450 2C5 [9] and *S. solfataricus* CYP119 [10]) will be released in 2001. Four of those currently accessible are for soluble bacterial enzymes. These are cytochrome *P*-450cam (CYP101; camphor hydroxylase from *Pseudomonas putida*) [11]; the heme domain from cytochrome *P*-450 BM3 (CYP102; a fatty acid hydroxylase from *Bacillus megaterium*) [12]; cytochrome *P*-450terp (CYP108; α-terpineol hydroxylase from a *Pseudomonas* species) [13]; and the substrate-bound form of *P*-450eryF (CYP107A1; 6-deoxyerythronolide B hydroxylase, involved in the synthesis of erythromycin biosynthesis in *Saccharopolyspora erythraea*) [14]. In addition, there are crystal structures of substrate-bound *P*-450cam and *P*-450 BM3 heme domain [15,16], as well as other ligand-bound structures for these enzymes. The fifth struc-

ture currently accessible is also a soluble enzyme, cytochrome *P*-450nor (CYP55A1), a nitric oxide reductase from *F. oxysporum* [6]. The remaining *P*-450 is *P*-450 sca2 from *Streptomyces carbophilus*, an industrially important enzyme which catalyzes the final hydroxylation step in the synthesis of the cholesterol lowering drug pravastatin [17]. All these enzymes display close structural similarity, at least in terms of overall topology, in spite of very low sequence identity. Generally, sequence identities are no more than 20%. The *P*-450 tertiary structure has an approximate trigonal prism arrangement containing both α-helical and β-sheet regions (Fig. 3). Helices dominate the structure, and many of these lie approximately parallel to the plane of the heme. The heme is bracketed by two helices: the I helix lies distal to the heme and spans the diameter of the protein structure, the shorter L helix lies proximal to the heme, and its N-terminus provides the cysteine-thiolate that proximally ligates the heme iron. The heme itself lies well buried within the structure, and the substrate binding site lies on its distal face. The substrate binding sites of the known *P*-450 structures are considerably different in size and shape, reflecting the nature of their substrates (Fig. 4). These differences are due to both topological variation in the polypeptide backbone and the nature of the side chains lining the active sites. In *P*-450 BM3, the long chain fatty acid substrate lies in an elongated hydrophobic channel that extends from the surface of the protein to the distal face of the heme [16]. By contrast, the substrate binding site of *P*-450cam is much deeper seated, and the substrate access channel is much less apparent. Comparison of substrate-bound *P*-450cam and *P*-450 BM3 structures with the respective substrate-free forms reveals conformational differences, demonstrating the importance of protein motion in allowing substrate access. The changes are particularly large for *P*-450 BM3. Furthermore, a crystal structure of *P*-450cam complexed with an imidazole ligating inhibitor considerably larger than camphor reveals the range of protein conformational lability and highlights the access route to the binding site [18]. The substrate binding site of *P*-450terp more closely resembles that of *P*-450cam, not unexpectedly given the similarity in substrate size, whilst that of *P*-450eryF is considerably larger. A further feature of *P*-450eryF is that in the substrate-bound form there is a network of hydrogen-bonded water molecules in the binding site. This is different from *P*-450cam and *P*-450 BM3 where water molecules are excluded.

The crystal structures for seven of the eight *P*-450s described above are of soluble enzymes that are readily overexpressed, purified and crystallized. The eukaryotic *P*-450s are membrane-bound and present major problems for X-ray structural determination. Until recently, no such crystal structure had been determined. Rational engineering of these enzymes has relied on sequence comparisons with bacterial enzymes whose structures had been determined and also on the generation of homology models. For the *P*-450 family 2, Gotoh [19] used sequence homology studies to identify six regions termed substrate recognition sites (SRS). These were based on the position of camphor in *P*-450cam, the only *P*-450 whose structure had been solved at the time. Subsequent



(a) D-camphor

(b) 6-deoxyerythronolide B

(c) α-terpineol

(d) palmitoleic acid

Fig. 4. Substrates for the cytochromes *P*-450 cam (a), eryF (b), terp (c) and BM3 (d).

protein engineering studies on *P*-450s 2A, 2B and 2C have validated this model for family 2 by showing that most residues identified as important for substrate specificity lie either within or near to these regions (e.g. [20–22]). The early homology models, based on the structure of *P*-450cam, include those generated for *P*-450s 1A1 [23], 2B1 [24] and scc (11A1) [25]. They suggested residues that could interact with the heme and substrate, or be involved in membrane attachment. A comparative study of the crystal structures of *P*-450cam, terp and BM3 and multiple sequence alignments, including *P*-450s from all the major families [26] suggested that all *P*-450s possess the same tertiary structure. Examples of recent molecular models generated include those for *P*-450 2A6 [27] and various members of the 4A subfamily [28] (based on the BM3 structure) and further models for *P*-450s 19A1 [29], 2B1 [30,31] (based on the structures of *P*-450s cam, BM3 and terp) and 3A4 [32] (based on the same three structures as well as that of eryF). Overall, the use of homology models of eukaryotic *P*-450s has facilitated both the elucidation of suitable candidates for mutagenesis and the interpretation of results of mutagenic studies. Conversely, results from site-directed mutagenesis studies have aided in the refinement and development of the models [33].

The recent determination of the structure of the first mammalian microsomal *P*-450 is a major breakthrough [9]. Membrane bound *P*-450s can be solubilized through modification of the protein by removal of the hydrophobic N-terminal anchor sequence, which is the major determinant of membrane binding (see examples in Section 3). The elegant studies of Johnson and co-workers have not only proven this for members of the mammalian 2C family, but have also shown that exchange of key residues from rabbit *P*-450 2C3 into *P*-450 2C5 generates highly soluble and monomeric *P*-450 progesterone hydroxylase [34]. This modified *P*-450 2C5 was crystallized and the structure solved to 3.0 Å [9]. There is remarkable similarity in the topology of this structure and those of the microbial *P*-450s, particularly with the *P*-450 BM3 structure (despite low amino acid sequence identity and different substrate selectivity). Key findings include identification of a broad hydrophobic surface likely to facilitate membrane interactions, and a putative substrate entry site located in this membrane attachment surface providing a direct substrate access/product exit route from the lipid bilayer.

2. Substrate specificity

The factors controlling the diversity in the substrate specificity of *P*-450s are still not fully understood. However, the availability of high resolution crystal structures for five of the microbial cytochromes *P*-450 [6,11–16] (see Section 1.1) has allowed the key active site residues that appear to be involved in substrate recognition to be identified. In the cases of cytochromes *P*-450 cam and BM3, the analysis of the crystal structures has been complemented by site-directed mutagenesis studies. This has enabled the precise roles of some of these residues to be elucidated, and provides the basis for active site re-design. Some of this work is outlined below. In addition, we also describe mutagenesis studies on *P*-450s 4A1 and 3A4 for which there are no crystal structures.

2.1. *Changing the substrate specificity of cytochrome P-450cam*

P-450cam specifically catalyzes the 5-*exo*-hydroxylation of camphor. The crystal structure of camphor-bound *P*-450cam [15] reveals a compact substrate binding region, surrounded by hydrophobic residues, lying distal to the heme (Fig. 5). The residues impor-

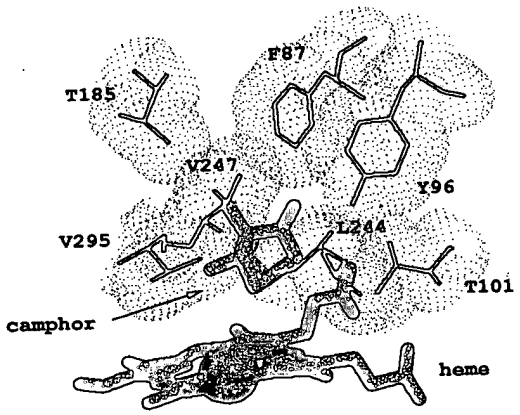


Fig. 5. The active site of *P*-450cam. Camphor and the heme cofactor are shown as thick lines, amino acid residues are shown as narrow lines with a dot surface.

tant for substrate binding and regio- and stereoselectivity lie within three tiers, approximately parallel to the heme plane. These include L244, V295 and T101 (Tier 1, closest to the heme) and Y96 and F87 (Tier 2), with Y96 being the only residue to make contact (H-bond) with the substrate [35]. A series of studies have been performed that employ protein engineering to probe factors pertaining to substrate specificity and the control of monooxygenation not only towards camphor, but also to substrates such as short chain alkanes and polycyclic aromatic hydrocarbons [36–38].

Work by Fowler et al. [38] and Stevenson et al. [36] involving mutagenesis of residue Y96 in *P*-450cam demonstrated how just one mutation can achieve a significant specificity change. Study of the Y96A mutant enzyme [38] showed that it favors the oxidation of diphenylmethane, a reaction not observed for the wild-type enzyme. A further change of this residue (Y96F) abolishes the strict regio- and stereospecificity observed for camphor hydroxylation. Furthermore, the Y96F mutation alters the specificity of *P*-450cam for polycyclic aromatic hydrocarbons such as naphthalene and pyrene [37]. With naphthalene, a high degree of regiospecificity was achieved, with 1- and 2-naphthol being predominant products (ratio 93:3, respectively). This shift in specificity was rationalized in terms of the increased active site volume caused by this mutation.

Stevenson et al. [36] examined the hydroxylation of alkanes such as pentane, hexane and 3-methyl pentane. Wild-type *P*-450cam hydroxylates these substrates at poor rates (k_{cat} values 0.9, 0.4 and 28 min^{-1}, respectively, c.f. approx. 300 min^{-1} for camphor hydroxylation), and with $\geq 80\%$ uncoupling (c.f., $< 5\%$ uncoupling for wild-type-catalyzed camphor hydroxylation). However, by increasing the hydrophobicity of the active site, turnover rates, as well as the degree of coupling, are markedly improved. The mutant enzyme Y96A hydroxylates pentane, hexane and 3-methyl pentane with turnover rates > 60 min^{-1}: Y96F is better still, with rates in excess of 100 min^{-1} and improved coupling. It was postulated that the increased hydrophobicity of the active sites in the mutants prevented protonation of the heme-bound oxygen in the ferric peroxide intermediate. The product profiles for both the wild-type- and the Y96 mutant-catalyzed oxidations are the same,

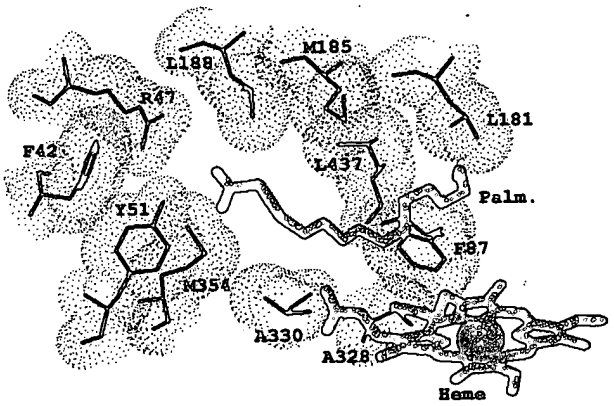


Fig. 6. The active site of *P*-450 BM3. Palmitoleate and the heme cofactor are shown as thick lines, amino acid residues are shown as narrow lines with a dot surface.

with tertiary hydroxylation predominating over secondary and primary. This is consistent with the general reactivity of CH bonds and implies that the substrate is mobile within the active site pocket. This would mean that the protein superstructure has little or no control over the orientation of these alkanes.

In more recent studies, the same group demonstrated that mutation of the surface residue F193 to alanine and isoleucine in a Y96F mutant background resulted in large decreases in catalytic rate with camphor, naphthalene and styrene [39]. F193 has been proposed to undergo dynamic fluctuations to permit substrate entry, and these data are consistent with structural changes that diminish catalytic potency of the enzyme.

2.2. *The roles of key active site residues in P-450 BM3*

Flavocytochrome *P*-450 BM3 hydroxylates a wide range of saturated and unsaturated fatty acids of chain length C_{12}-C_{20} at the ω-1 to ω-3 positions. In contrast to the compact active site of *P*-450cam, that of BM3 [16] consists of a long, hydrophobic channel, extending from the distal face of the heme to the protein surface (Fig. 6). In addition to the hydrophobic residues lining the site, comparisons of the substrate-free and palmitoleate-bound crystal structures have indicated that other residues are important in protein-substrate interactions. At the mouth of the active site lies R47 (which provides an electrostatic interaction with the carboxylate end of the

fatty acid) and Y51 (which can provide a hydrogen bond to the carboxylate). One further notable residue at the mouth of the active site, F42, appears to cover the opening to the active site channel, providing what might be termed a hydrophobic 'lid'. At the opposite end of the active site channel, close to the heme, lies the side chain of F87. The importance of this residue was highlighted by the large conformational difference adopted by the phenyl ring in the substrate-free and palmitoleate-bound crystal structures [12,16]. In the substrate-free form the side chain lies perpendicular to the heme plane but in the palmitoleate-bound form it has twisted to lie almost parallel to it. In this section we describe the site-directed mutagenesis studies that have defined roles for these residues in substrate binding and in the control of regiospecificity and altered substrate specificity.

The important contribution of the guanidinium group of R47 has been confirmed by mutagenesis experiments. In a study by Oliver et al. [40] the substrate binding characteristics of wild-type *P*-450 BM3 and the mutant R47E and their abilities to catalyze substrate oxidation were compared. The mutant enzyme retained activity towards C_{12}-C_{16} fatty acids (but with k_{cat}/K_m values 14–21-fold lower than wild-type), as well as the regioselectivity of oxidation (ω-1 to ω-3). However, unlike the wild-type enzyme, the R47E mutant was able to efficiently hydroxylate C_{12}-C_{16} alkylammonium compounds (also at the ω-1 to ω-3 positions), with k_{cat} values of up to 19 s^{-1}. Optical spectroscopy showed that fatty acid binding to the mutant is much weaker than to the wild-type enzyme, and paramagnetic relaxation experiments showed that the fatty acid adopted a different orientation in the active site channel. In a further study, Noble et al. [41] demonstrated the dominance of the side chain of R47 over that of Y51 in providing the major interaction with the fatty acid carboxylate. The same study highlighted the importance of the phenyl side chain of F42 in providing a hydrophobic 'lid' to the active site (K_m values were increased almost 10-fold for laurate and arachidonate turnover). This latter phenomenon was interpreted as the consequence of ingress of water molecules to the active site and the disruption of the strong ion pair interaction between the R47-guanidino group and the fatty acid carboxylate.

Phenylalanine 87 is located at the base of the active site of *P*-450 BM3 and, from inspection of the palmitoleate-bound *P*-450 BM3 crystal structure, appears to protect the terminus of the fatty acid from ω-hydroxylation. Consistent with this, substitution of F87 by alanine converts *P*-450 BM3 from a fatty acid hydroxylase that oxidizes at the ω-1 to ω-3 positions to one that oxidizes at the ω position [42] (a reaction not observed for wild-type *P*-450 BM3 but common to many mammalian *P*-450s such as those belonging to the 4A1 family). In the F87A mutant enzyme a decrease in K_d for laurate of up to 6-fold was observed, showing that the removal of the bulky phenyl side chain facilitates tighter binding of medium chain (e.g. C_{12}) fatty acids. However, the K_m for laurate oxidation catalyzed by the F87A mutant was found to be identical to that seen for the wild-type enzyme. This was suggested to be due to a difference in the substrate binding to the ferrous and ferric forms. The implication is that the F87A mutation causes a change in the substrate binding, not in the initial Michaelis complex, but in a subsequent step in the catalytic cycle. Proton NMR experiments have shown that laurate and 12-bromolaurate bound in the active sites of wild-type and F87A *P*-450 BM3 move some 6 Å closer to the heme upon iron reduction [43]. Molecular modeling studies indicate that the absence of the phenyl ring enables the ω-terminus of laurate to approach closer to the heme in the F87A mutant than in the wild-type enzyme, and these data were supported by NMR studies (ω-CH_3···Fe = 5.1 Å in wild-type and 3.1 Å in the F87A mutant enzyme). This explains the mixed product distribution observed in wild-type for laurate hydroxylation and the almost exclusive (>90%) ω-terminal hydroxylation demonstrated by the F87A mutant.

2.3. *Modification of the regiospecificity of cytochrome P-450 4A1*

P-450s of the 4A family, found in the liver and kidneys of mammals, are long chain fatty acid monooxygenases that predominantly hydroxylate at the ω position. Of these, *P*-450 4A1 is the most extensively studied [44]. A possible explanation for the strict ω-terminal specificity is that the active site of *P*-450 4A1 is highly constrained, so that substrate binding orientation is precisely defined. The specific-

ity of this enzyme for a range of fatty acid chain lengths, such as laurate (C_{12}), palmitate (C_{16}) and arachidonate (C_{20}), suggests a possible structural similarity with *P*-450 BM3. Hence, by sequence alignment of *P*-450s 4A1 and BM3 (considered as the closest structurally defined model of the 4A family), two residues of 4A1, E320 and D323, were identified as structural equivalents of A264 and E267 in *P*-450 BM3. In the crystal structure of palmitoleate-bound *P*-450 BM3, these two residues lie close to the ω-terminus of the fatty acid tail. To establish the importance of these residues in *P*-450 4A1, the reciprocal mutants E320A, D323E and the double mutant E320A/D323E were constructed. For all three mutant *P*-450s, there was a relaxation of the regiospecific hydroxylation of laurate: in wild-type *P*-450 4A1 the ω:ω-1 product ratio is 20:1. However, in the E320A/D323E double mutant enzyme this ratio drops to only 2:1. The NADPH consumption rate in the reconstituted *P*-450 mutant system did not vary from that observed for the wild-type (approx. 300 min^{-1}), but significantly lower laurate oxidation rates for the mutant-catalyzed reactions (<40 min^{-1} for all three mutants, compared to 150 min^{-1} for wild-type) were found, which indicates significant uncoupling. The conclusion is that the two residues, E320 and D323, are located in the active site and are involved in substrate binding. At present there is no experimental evidence for key roles for either A264 or E267 in *P*-450 BM3 substrate binding or catalysis. It would be interesting to see whether the reciprocal mutations in *P*-450 BM3 (A264E and E267D) would confer ω-hydroxylation activity.

2.4. *Identification of key residues in P-450 3A4 involved in substrate and effector binding*

Family 3 of the cytochromes *P*-450 consists of just one subfamily, *P*-450 3A [45], which plays a major role in hepatic biotransformation pathways. Human 3A4 is the most abundant *P*-450 in the liver (up to approx. 60% of total *P*-450 content in some cases) and metabolizes a wide range of clinically important drugs [46] in addition to steroids [47] and carcinogens [48]. Its importance is underlined by the fact that it is reported to be involved in the metabolism of around 50% of currently used drugs [46,49]. *P*-450 3A4 exhibits cooperativity towards some substrates; for example, homotropic cooperativity with progesterone [50] and testosterone [51]. Activity can also be influenced heterotropically, as in the stimulation of progesterone and testosterone oxidation by the effector α-naphthoflavone (ANF) [50].

Rational protein engineering studies on *P*-450 3A4, to study, for example, the activation by ANF (several proposed mechanisms are outlined in [52]) or substrate specificity, are potentially obstructed by the absence of its crystal structure and the fact that there is little pronounced variation in substrate specificity for the 3A enzymes (in contrast to family 2, for example) [52]. However, the construction of homology models of 3A4 has greatly aided determination of candidates for mutagenesis. One structural model was based on amino acid sequence alignments with *P*-450 BM3 and a number of substrate and inhibitor interactions were investigated [53]. Another model was built using consensus modeling methods to base the structure on those of *P*-450s cam, BM3, terp and eryF [32]. Although this model bore most resemblance to BM3, the use of the eryF structure was reported to be important in defining the size of the active site through localization of the B′ helix. Progesterone or erythromycin were docked at the active site and the potential contact residues noted.

The first site-directed mutagenesis study was carried out on *P*-450 3A4 using alanine scanning mutagenesis to probe the roles of residues 210–216 [52]. (It also indicated that Gotoh's substrate recognition site model was applicable to the *P*-450s 3A.) Mutant enzymes L210A and L211A led to a decrease in stimulation of testosterone and progesterone hydroxylation effected by ANF and changes in the regioselectivity of testosterone hydroxylation were also observed with L210A. This indicated that substrate and effector binding sites could be partially overlapping. To test this a double mutant was constructed in which L211 and D214 (the latter chosen after study of the Szklarz model [32]) were changed to phenylalanine and glutamic acid, respectively [54]. The idea was to mimic effector binding by narrowing the sizeable binding pocket, considered large enough to be able to accommodate both a steroid and ANF at the same time (e.g. [32]), through the introduction of bulkier residues. As a consequence, homotropic cooperativity for steroid hydroxylation was eliminated and a reduced response to stimulation by ANF was

observed. Further analysis of the model for residues that could affect cooperativity suggested that F304 (positioned on the highly conserved I helix and suggested experimentally [55] and from the model to play a role in substrate binding) was a likely candidate [56]. As with the L211F/D214E double mutant, the F304W enzyme was unable to fully mimic effector binding. However, the triple mutant L211F/D214E/F304W was much more effective.

Other residues whose mutation influenced stimulation of activity by ANF are I369, L373 (SRS 5) [57] and A305 (SRS 4) [55].

Related studies of *P*-450 3A4 probed aspects of substrate binding and specificity. It was shown through mutagenesis of S119 from SRS 1, a residue predicted from modeling to be involved in progesterone binding [32], that *P*-450 3A4 could be converted from a steroid 6β-hydroxylase to a 2β-hydroxylase [58]. This residue may occupy a similar position to F87 in *P*-450 BM3 [32,58], shown to be important in controlling regioselectivity of substrate oxidation (see Section 2.2). Cassette and site-directed mutagenesis were carried out on amino acids 364–377 in SRS 5 [57]. Two of these residues, 370 and 373, were predicted from the model to interact with progesterone when the substrate was docked in the 6β-orientation [32]. Mutant enzymes I369V, A370V and L373H all exhibited altered progesterone metabolic profiles. I369V and A370V showed decreased and increased 16α-hydroxylation activities, respectively, compared to the wild-type enzyme. However, mutant L373H, whilst retaining progesterone 6β and 16α activities, produced a new, unidentified, metabolite. Analysis of progesterone docked in the 16α binding orientation in the model suggested that the effects observed with the I369V and A370V enzymes (involving substitution with a smaller and a larger residue, respectively) were due to changes in substrate mobility within the active site.

In addition to the above studies to probe substrate and effector binding, the structure-function relationship between *P*-450s 3A4 and 3A5 was investigated using differences in the regiospecificities of the two enzymes (which share >85% amino acid sequence identity) towards the biotransformation of the carcinogen aflatoxin B_1 [59]. The six, putative substrate recognition sites of the two enzymes were compared and non-conserved residues between the two enzymes were replaced in *P*-450 3A4 with those of *P*-450 3A5. Two, individual, mutations in SRS 2 (N206S and L210F) had the greatest effect, conferring the phenotype of the 3A5 enzyme onto *P*-450 3A4.

Thus far, analysis of *P*-450 3A4 through the use of homology models and mutagenesis in the absence of a crystal structure has provided significant advances in the understanding of substrate and effector binding in this enzyme. Eventually it is hoped that these and further studies will allow prediction of drug/drug interactions with *P*-450 3A4 [56].

2.5. *Chimeragenesis as a probe of substrate specificity*

The absence (until recently) of crystal structures for mammalian *P*-450s limits solid understanding of the factors that confer substrate specificity. However, one approach to this problem that has proved successful is the use of chimeric constructs in combination with site-directed mutagenesis to identify structural determinants of substrate specificity within a *P*-450 subfamily. In most cases, two *P*-450s with high sequence identity but different substrate specificities have been investigated by substituting a region of one with the corresponding region from the other and analyzing for any change in substrate specificity. Once regions of interest have been elucidated, site-directed mutagenesis can be used to pinpoint key individual residues that are responsible for the particular substrate specificity. A number of such studies using *P*-450 chimeras have been published over the last 5 years, the majority concerning *P*-450 family 2. These include: 2C1 and 2C2 [60]; 2B1 and 2B2 [61]; 2B4 and 2B5 [62]; 2C9 and 2C19 [63–65]. For this reason, we have chosen to highlight this family in this section of the review, focusing on human *P*-450s 2C9 and 2C19. Other examples discussed are canine *P*-450s 3A12 and 3A26 [66] and *P*-450s Cm2 and Alk3A from the yeast *Candida maltosa* [67]. These studies demonstrate the power of the chimeragenesis approach in elucidating the controlling factors of substrate specificity.

2.5.1. *Probing substrate specificity of cytochromes P-450 2C9 and 2C19*

The *P*-450s of the 2C family comprise approx. 16% of the total hepatic *P*-450 content [68]. They are weakly inducible by phenobarbital, and are fre-

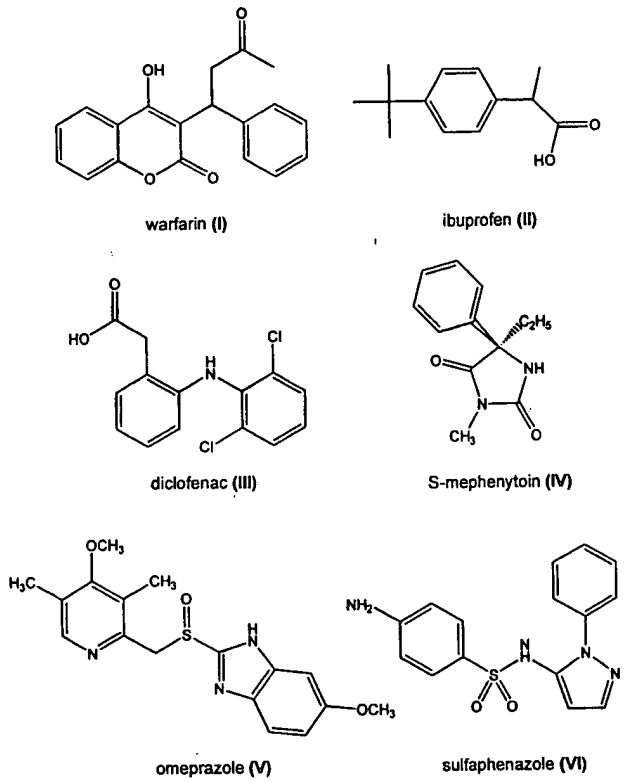


Fig. 7. Substrates for *P*-450s 2C9 and/or 2C19.

quently involved in hydroxylations of aromatic ring systems. Endogenous substrates for the 2C subfamily are usually steroids, although rabbit *P*-450 2C2 is known to hydroxylate laurate at the ω-1 position. Human *P*-450s 2C9 and 2C19 are closely related, with 92% sequence identity [69]. 2C9 is a testosterone 16β-hydroxylase, but the physiological substrate for 2C19 is not clear. Both metabolize a number of clinically important drugs, an example common to both being the anti-coagulant warfarin (Fig. 7, I) [65]. However, the regio- and stereospecificities of 2C9 and 2C19 towards this substrate differ. The non-steroidal, anti-inflammatory drugs ibuprofen (II) and diclofenac (III) are principally metabolized by 2C9 [70]. 2C19 does not metabolize either of these compounds [71,72], but shows selectivity for 4′-hydroxylation of the anti-convulsant *S*-mephenytoin (IV) [73] and 5′-hydroxylation of omeprazole (V) [74] (an anti-ulcer drug). In general, 2C19 appears to have less stringent steric requirements than does 2C9 [65]. A further difference between the two is that 2C9 is inhibited by sulfaphenazole (VI) (K_d < 1 μM) whereas 2C19 is not inhibited by this compound at concentrations even up to 100 μM [75,76]. A detailed knowledge of the determinants of substrate specificity in these two *P*-450s could provide the basis for the design of drugs less dependent on metabolism by a specific isoform [65]. In other words, their metabolism would be less affected by genetic variation, an important factor considering, for example, that 15–20% of Orientals and 2–5% of Caucasians do not express 2C19 [77].

Using the substrate recognitions sites identified by Gotoh [19], Ibeanu et al. [64] constructed 2C9/2C19 chimeras and site-directed mutant forms of 2C9 to elucidate the key determinants of omeprazole hydroxylation by 2C19. A 2C9 chimera containing residues 160–227 from 2C19 in combination with the point mutation I99H gives an engineered *P*-450 2C9 which displays greater omeprazole 5-hydroxylase activity than does 2C19. Further site-directed mutagenesis showed that three residues from 2C19 were found to confer omeprazole activity into 2C9. The I99H, S220T and P221T triple mutant of 2C9 hydroxylated omeprazole as efficiently as 2C19. Residue 99 is located within a predicted substrate recognition site (SRS 1) whereas 220 and 221 are not. According to the results of sequence analysis with *P*-450cam they are located on the turn between the F and G helices [19]. This region varies considerably in length between *P*-450s and may form a flexible cap over the active site, thereby forming part of the substrate access channel [26,64,78].

More recent studies [63] have determined the critical areas of *P*-450 2C9 which define the specificity for diclofenac and ibuprofen. Chimeras of both 2C9 and 2C19 were constructed with the emphasis on SRS 3 and 4. Replacing residues 228–340, containing SRS 3 and 4, of 2C19 with those of 2C9, generated a chimera that could hydroxylate both drugs. Interestingly, the regiospecificity for ibuprofen hydroxylation differs from that of wild-type 2C9, probably indicating that the orientations of the bound substrate are slightly different. The converse construct had little or no activity towards diclofenac or ibuprofen. Individual substitution of SRS 3 and 4 revealed that the latter was the most important region for conferring specificity. As the two isoforms differ in only five residues within SRS 4 (283–340), site-directed mutagenesis was carried out on a 2C19 chimera contain-

ing residues 228–282 of 2C9 (including SRS 3) to pinpoint the key residue(s). These studies showed that the I289N mutation confers specificity for diclofenac hydroxylation. The activity increased when a second mutation, N286S, was introduced. In addition it was shown that S286 in combination with N289 confers specificity for ibuprofen, the former residue appearing to be the major contributor. Both these residues are found in a highly conserved region corresponding to the I helix of structurally characterized cytochromes *P*-450.

The importance of residues N289 and S286 in *P*-450 2C9 was again underlined by Jung et al. [65] who investigated the amino acid residues that confer a high affinity for sulfaphenazole binding and a high catalytic efficiency for warfarin metabolism. Two substitutions of 2C9 residues into 2C19, N286S and I289N, were found to be sufficient to confer high affinity binding of the inhibitor sulfaphenazole ($K_d = 4$ μM). Interpretation of results was facilitated by the generation of a homology model of 2C9 with docked sulfaphenazole which indicated that it was unlikely that S286 and N289 contacted the inhibitor. However, it was considered that these residues could influence substrate access or active site packing. In combination with E241K (mutation of a residue located in the region corresponding to helix G), the 2C19 double mutant was able to hydroxylate both *R* and *S* enantiomers of warfarin with a higher catalytic efficiency than observed for wild-type 2C19 [65].

2.5.2. *Substrate specificity in cytochromes P-450 3A12 and 3A26*

Chimeragenesis has been used to determine residues responsible for differences in steroid hydroxylation catalyzed by two canine *P*-450s, 3A12 and 3A26 [66]. Although these enzymes have 96% amino acid sequence identity (differing by only 22 of 503 residues [45]) the former exhibits higher rates of steroid hydroxylation. The approach used was to monitor the loss of progesterone 6β-hydroxylation activity in *P*-450 3A12 caused by the introduction of sequences from 3A26. Once regions responsible for the loss of activity had been identified, site-directed mutagenesis of non-identical residues within such regions was employed to restore activity of the chimera back to that of *P*-450 3A12. Three residues, at positions 187, 368 and 369 were identified as those mainly responsible for the differences in steroid 6β-hydroxylation between the two proteins. When these three were mutated in *P*-450 3A26 to those of 3A12 (I187T, S368P and V369I), a 10–20-fold increase in the ability of 3A26 to hydroxylate steroids was observed. All the testosterone and androstenedione 6β-hydroxylase activity of 3A12 was conferred to 3A26. These canine cytochromes *P*-450 are considered as models for human drug metabolism since residues identified in this study are also important for the function of human *P*-450 3A4 [57,66].

2.5.3. *The substrate specificities of cytochromes P-450 Cm2 and Alk3A*

Cytochromes *P*-450 Cm2 and Alk3A are CYP52A4 variants naturally occurring in the yeast *C. maltosa* [79]. The family 52 *P*-450s are alkane and fatty acid hydroxylases related to the mammalian *P*-450 4A and the bacterial *P*-450 BM3 (around 25% amino acid identity) [80]. Zimmer et al. [80] used chimeragenesis to transpose the substrate specificities of cytochromes *P*-450 Cm2 and Alk3A. Although both these enzymes show affinities for laurate and palmitate, *P*-450 Cm2 displays greater efficiency for the ω-hydroxylation of laurate. Five pairs of chimeric constructs were devised in order to investigate each of the seven amino acid differences between the two enzymes. The key residue was found to be at position 527 (a valine in *P*-450 Cm2 and a leucine in Alk3A). Analysis of an engineered enzyme pair in which V527 of Cm2 and L537 of Alk3A were exchanged showed a transposition of specificity, providing compelling evidence that position 527 was the key residue modulating fatty acid turnover. To show that this residue was acting independently it was changed to methionine in both *P*-450s to give enzymes with almost identical activities (more similar to that of Cm2). The fact that such subtle differences in side chain lengths between valine and leucine could have such effects suggested that residue 527 must be at the active site, and may even play a role in holding the terminal methyl group of the substrate at the correct position for monooxygenation.

In addition to this 'directed' chimeragenesis, an alternative random chimeragenesis approach [81,82] has been used in an attempt to map the active sites of

the distantly related *P*-450s scc and c27 [83]. It was concluded from this work that an amino acid identity of 25% or greater between *P*-450s is necessary for the successful application of this technique [83]. A more recent study used random chimeragenesis to probe species-specific biochemical differences between rat and human *P*-450c17, two proteins with 68% amino acid sequence identity [84]. However, the five chimeras generated did not exhibit activity patterns that equated to those of the parents, limiting the amount of useful structure/function information that could be obtained.

2.5.4. *Bacterial/mammalian chimeric cytochromes P-450*

To examine whether mammalian and bacterial enzymes are structurally related and if the bacterial structures provide a valid basis for building homology models of mammalian enzymes, Shimoji et al. [85] constructed a novel chimera from the cytosolic bacterial cytochrome *P*-450cam and the membrane-bound mammalian cytochrome *P*-450 2C9. These two cytochromes have less than 15% amino acid sequence identity. To ensure solubility the chimera was designed with the N-terminal 216 amino acids of *P*-450cam fused to a fragment of *P*-450 2C9 from residue 257 to the C-terminus. This accommodated the putative substrate recognition sites 1–3 of *P*-450cam and 4–6 of *P*-450 2C9. No mammalian amino acids prior to the random coil between the G- and H-helices were included to avoid any potential membrane interactions. The resultant chimera was soluble and correctly folded. Assays of catalytic activity (the oxidation of 4-chlorotoluene to 4-chlorobenzyl alcohol) demonstrated that the chimera was functionally active. The fact that the chimera folded correctly was considered indicative of conservation between the three-dimensional structures of these bacterial and mammalian *P*-450s. A further application suggested for such chimeragenesis was the construction of soluble *P*-450s with active site characteristics of mammalian forms, which could prove of use in crystallization studies and bioremediation [85]. Also of interest would be to further characterize such chimeras with different reductase components to delineate regions important in the two enzymes for protein/protein interactions and electron transfer.

3. Protein engineering to alter membrane binding

With the exception of *P*-450nor, all eukaryotic *P*-450s identified are membrane-bound. For the microsomal *P*-450s, which are bound to the endoplasmic reticulum (ER), the major determinant of membrane binding seems to be a hydrophobic stretch of amino acids at the N-terminus. The remaining polypeptide folds into a globular form that resides above the membrane. In most microsomal *P*-450s the N-terminal region contains a sequence of 16–20 non-polar side chains that are believed to provide the major hydrophobic interaction with the ER membrane. When the first 29 N-terminal residues of cytochrome *P*-450 2C1 were fused to a globular secretory protein (β-galactosidase), or to a soluble cytosolic protein (alkaline phosphatase) the resulting polypeptide was translocated to the ER [86]. Experiments on cytochrome *P*-450 21B have shown that removal of more than one third of the hydrophobic residues in the N-terminus result in the production of a cytosolic *P*-450 form [87]. Such studies demonstrate that N-terminal deletion or modification could facilitate heterologous expression of the *P*-450s, for the overproduction of soluble forms of microsomal *P*-450s for various applications. However, the overexpression of N-terminally truncated forms of several *P*-450s in *Escherichia coli* has shown that both the yield of soluble protein and the catalytic activity are highly variable and dependent on the *P*-450 in question [88]. For example, full-length *P*-450s 2E1 and 2B4 are both targeted to the bacterial inner membrane, but whereas deletion of residues 3–29 from 2E1 still locates the protein predominantly in the membrane fraction (65%), deletion of residues 2–20 from 2B4 locates 68% of the protein to the cytosol. Fusion of the N-termini from 2E1 and 2B4 to the heme domain of the soluble bacterial *P*-450 BM3 had the effect, in both cases, of locating only 20 and 27%, respectively, to the bacterial membrane fraction [89]. This indicates that there are different determinants of membrane binding between 2E1 and 2B4 on the globular domain distinct from the N-terminal anchor. Thus, the N-terminal portion of the eukaryotic *P*-450s is not the sole determinant of membrane binding. Further, isoform-specific, structural motifs on the surface of the globular *P*-450 domain must interact with the membrane. It has

been demonstrated by chemical labeling [90] and mutagenesis [91] experiments that sites even close to the C-terminus may be involved in membrane interactions.

Although recombinant eukaryotic *P*-450s often need to be purified in the presence of detergents (even after N-terminal modification) this is not always the case. For example, Sueyoshi et al. [92] were able to purify a modified form of *P*-450 2A4 from *E. coli* without the use of detergents. The hydrophobic N-terminal residues 2–19 were substituted for a sequence that forms an amphipathic helix, and although the modified form of the protein was initially localized to the membrane fraction, it could be readily freed by treatment with sodium carbonate. In another example a soluble form of cytochrome *P*-450 51 (*P*-450 sterol 14α-demethylase) from *Candida albicans* has been engineered with a modified N-terminus containing a protease cleavage site [93]. Initial solubilization using detergent can be followed, after the cleavage reaction, by further purification without the use of detergent.

The recent breakthroughs in the expression of soluble domains of eukaryotic *P*-450s and *P*-450 reductases without their N-terminal membrane spanning domains have provided great hope that more crystal structures can be solved for mammalian *P*-450 systems. In addition, it has been shown that soluble *P*-450 domains heterologously expressed in *E. coli* can retain activity, and that this activity can be supported by the host enzymes flavodoxin and $NADP^+$-flavodoxin oxidoreductase [94]. This has raised expectations that *E. coli* expressing different human *P*-450 isoforms and utilizing either the host redox system or co-expressed human *P*-450 reductase, can be used as a model to predict the metabolic fate of drugs and identify the *P*-450s responsible [95]. Yeast (*Saccharomyces cerevisiae*) is another attractive system for simultaneous expression of multiple human *P*-450s [96]. Systems of this type have great potential in mutagenicity testing and predictive metabolism [97].

Protein engineering studies to promote solubility of a *P*-450 and to prevent its oligomerization have seen their greatest success only this year. After intensive studies of rabbit progesterone hydroxylase *P*-450s 2C3 and 2C5, Eric Johnson and co-workers were able to generate a modified *P*-450 2C5 that could be expressed in a soluble, monomeric form in *E. coli*. In initial studies, these workers showed that by deletion of the regions encoding the N-terminal membrane spanning sections and by incorporation of a 4-histidine tag at the proteins' C-terminus, the *P*-450s could be overexpressed in *E. coli* and solubilized from the membrane by high salt [34]. The *P*-450s could then be purified without use of detergents. However, the 2C3 and 2C5 enzymes formed dimers and tetramers, respectively, and these forms could not be crystallized. Subsequent studies of *P*-450 2C3/2C5 chimeras showed that replacement of selected amino acids in *P*-450 2C5 with those from 2C3 resulted in production of *P*-450 2C5 hybrids that retained catalytic activity and remained monomeric after solubilizing with salt [98]. The monomeric *P*-450 2C5 enzyme containing five substitutions from 2C3 (N202H, R206E, I270L, S209G and S210T) was crystallized successfully, leading to the determination of the first atomic structure of a mammalian *P*-450 [9].

4. Electron transfer

4.1. A thermodynamic switch

For the well-characterized bacterial *P*-450s cam and BM3, the binding of substrate (camphor and fatty acid, respectively) displaces the sixth heme ligand (water) and shifts the ferric heme iron from low to high spin. This results in a positive shift in heme reduction potential of some 130 mV. The magnitude of this change makes electron transfer from a redox partner thermodynamically favored [99,100]. The biological benefit of this thermodynamic 'switch' in electron transfer is an avoidance of the futile production of oxygen radicals and/or hydrogen peroxide in the absence of substrate. However, such control does not appear to be operative for all *P*-450s.

4.2. Probing the route of electron transfer

The analysis of the route of electron transfer in the *P*-450s has been intensively studied since the determination of the first *P*-450 crystal structure, *P*-450cam [11]. In this class I system (Fig. 1), NADH reduces the FAD containing putidaredoxin

reductase (PdR), which shuttles electrons one at a time through the iron-sulfur containing putidaredoxin (Pd) to the *P*-450 [2]. The system is analogous to the eukaryotic mitochondrial class I *P*-450s, such as that for *P*-450scc (partnered by adrenodoxin and adrenodoxin reductase) [101]. In this system, the interactions between the various redox components are thought to be mainly electrostatic [102,103]. Electrostatics are also thought to dominate redox partner binding in *P*-450cam and other class I systems. Stayton and co-workers defined the Pd docking site on *P*-450cam [104]. At least four basic residues on the proximal side of the *P*-450cam heme iron (R72, K344, R112, Q343 and, perhaps, R364) are involved in the interaction with residues of opposite charge on Pd (D58, E65, E67 and E72). It has been postulated that the three components of these class I *P*-450 systems can come together as a ternary complex for catalysis [105]. However, a more realistic model for the class I systems may be that the exposed iron-sulfur centers of the redoxin proteins shuttle electrons singly between the reductase and the *P*-450. The reaction between putidaredoxin and the *P*-450 is among the best studied of all biological electron transfer reactions. The first and second electron transfers from putidaredoxin to the heme iron are the two slowest steps in catalysis. At physiological concentrations of Pd, the second electron transfer is the overall rate limiting step [106]. The decrease in apparent affinity (elevated K_m value) for these redox partners observed with increasing ionic strength is consistent with an electrostatic interaction. However, what appears to be a straightforward set of electrostatic interactions between redox partners is in fact much more complex. This is demonstrated by the fact that *P*-450cam has completely different affinities for oxidized and reduced putidaredoxin [99,107]. The C-terminal tryptophan of putidaredoxin has been found to be a major determinant for the higher affinity for reduced Pd [108]. Much recent attention has focused on the role of arginine 112 in *P*-450cam – a surface residue which is hydrogen bonded to a heme propionate. Substitution to various neutral residues resulted in decreased electron transfer rates to the heme iron, and resulted in more negative heme reduction potentials. Mutation R112K had a less severe effect, but did show similar trends in reduction rate and heme potential [109]. These data are consistent with the studies of Nakamura and co-workers, who also showed that heme content and *P*-450 stability were affected by mutation at this residue (R112Q and R112E mutant enzymes) [107]. Other recent studies on the *P*-450cam system have implicated Pd residue E72 in the stabilization of the Pd/PdR complex, and Pd residue V98 in hydrophobic interactions with *P*-450cam [110,111]. Through the application of electrostatic and electron transfer theories, and taking into account prior mutagenesis results, Roitberg and co-workers proposed an electron transfer pathway from the iron sulfur center of Pd to the heme of *P*-450cam involving R38 and C39 on Pd, and R112 on the *P*-450 as a conduit to the heme propionate group [112].

It appears that redox partner docking is similar in the prokaryotic and mammalian systems, in the sense that all interact with either ferredoxin or *P*-450 reductase partners and since the site of docking is at the proximal face of the heme. However, strong evidence for direct mediation of electron transfer by amino acid side chains has not been found. The involvement of a tryptophan at the C-terminus of putidaredoxin (W106) in electron transfer was considered likely, based on the fact that its removal led to a large decrease in the rate of electron transfer [113]. Furthermore, a highly conserved tryptophan residue throughout the *P*-450 enzyme superfamily, the equivalent to W96 in *P*-450 BM3, for example, is notably absent from *P*-450cam. It was therefore postulated that this aromatic residue is an obligatory electron transfer mediator in the *P*-450s, and that the *P*-450cam system is an unusual example in which the tryptophan is a component of the redox partner instead [114]. The role of W96 in *P*-450 BM3 was investigated, and it was found that mutant enzymes W96A, W96Y and W96F bound sub-stoichiometric amounts of heme, but were otherwise catalytically competent [115]. The subsequent solution of the crystal structure of the *P*-450 BM3 heme domain indicated that W96 is in hydrogen bonding distance to a heme propionate group [12], helping to explain the effects on heme content. In other studies on the *P*-450cam system, it was found that the removal of W106 from putidaredoxin resulted in a large increase in the K_d of the reduced form for *P*-450cam [108]. Thus the tryptophan residues in the *P*-450cam and *P*-450 BM3 systems are required for efficient redox

partner interaction and heme binding, respectively. A direct role in electron transfer has yet to be proven.

4.3. The role of cytochrome b_5

Cytochrome b_5 is involved in many mammalian *P*-450 reactions, particularly those catalyzing steps in steroid metabolism. It may donate the second electron to the oxyferrous *P*-450 during catalysis, but is known to act as a structural effector of *P*-450 function – as evidenced by the ability of apo (heme-free) b_5 to stimulate activity of cytochrome *P*-450 3A4 [116]. Also, both holo- and apo-cytochrome b_5 augment the reaction profile of the human cytochrome *P*-450c17 system in favor of the 17,20-lyase reaction (cleavage of the C_{17}-C_{20} bond), while the b_5-free enzyme system favors 17α-hydroxylation [117]. These data indicate that b_5 has allosteric roles, but redox functions may also be operative and the precise role is probably dependent on the *P*-450 system involved. Cytochrome b_5 binding frequently perturbs the *P*-450 heme iron spin state equilibrium in favor of the high spin form, in similar fashion to the binding of substrates. However, its binding site is on the distal, not the proximal, face of the heme. Microsomal cytochrome b_5 has a highly charged surface and is also thought to bind electrostatically to *P*-450s, with carboxylate groups important to the interaction. In elegant experiments, Stayton and co-workers modified bovine cytochrome b_5 by replacement of surface threonines with cysteines. This enabled the attachment (via the sulfhydryl groups) of the fluorophore acrylodan and the use of fluorescence as a tool to measure the binding of b_5 to *P*-450cam. A K_d value of 1.1 μM was thus determined, and Pd was shown to competitively inhibit the binding of b_5 [104,118]. These results indicate that both proteins bind at overlapping sites on the distal face of *P*-450cam [119]. The fact that cytochrome b_5 binds tightly to *P*-450cam is indicative that the bacterial cytochromes *P*-450 retain strong structural similarity to their mammalian counterparts. The incisive *P*-450 2B4 mutagenesis studies of Waskell and co-workers have helped to define the binding site for cytochrome b_5, demonstrating the importance of the *P*-450 C- and C′-helices in the b_5 interaction site [120]. There is extensive, but not complete, overlap of the *P*-450 reductase and b_5 binding sites on the proximal surface of the *P*-450.

Until recently, the interaction between cytochrome b_5 and bacterial *P*-450s (*P*-450 BM3 also binds b_5) was considered to reflect merely an evolutionary link between redox partner binding sites in prokaryotic and eukaryotic *P*-450s, since b_5 was thought to be exclusively a eukaryotic redox protein. However, recent studies indicate that bacteria may also encode cytochromes b_5, indeed the first three-dimensional structure of a bacterial cytochrome b_5 has been reported [121]. It may be the case, then, that certain bacterial *P*-450s are also able to use b_5 as a redox partner.

4.4. Flavocytochrome P-450 BM3

The soluble flavocytochrome *P*-450 BM3 system is considered the best model for deconvoluting electron transfer in class II *P*-450 systems [122]. Genetic dissection of the enzyme has allowed the expression of its component domains. Initially, the *P*-450 domain (residues 1–472) and the diflavin reductase domain (residues 472–1048) were expressed independently in *E. coli* [123]. More recently, the subdomains of the diflavin domain have been overproduced in *E. coli*. These subdomains are ferredoxin reductase-like (residues 654–1048) and flavodoxin-like (residues 471–664), each binding a single flavin cofactor [124]. The ability to produce proteins with single redox cofactors has enabled the deconvolution of their otherwise overlapping spectral properties, and the determination of the reduction potentials for all the cofactors [124]. This study revealed that fatty acid substrate binding to the *P*-450 resulted in an approx. 130 mV increase in the reduction potential of the heme iron (from −368 to −239 mV on arachidonate binding), triggering electron transfer from the FMN (see Section 4.1).

The electron transfer pathway in *P*-450 BM3 has not been as intensively studied as that in *P*-450cam, although W96 in the heme domain has been ruled out as an electron transfer mediator [115]. In studies of the reductase domain of the enzyme, Klein and Fulco demonstrated that W574 affected electron transport in the enzyme [125]. However, these defects may be explained by low incorporation of FMN, and

possibly by perturbation of the reduction potentials of the FMN. Recently, the crystal structure of the combined FMN and heme domains has been reported [126]. Unfortunately, the FMN domain of the protein was found to be proteolytically separated from the heme domain, and the stoichiometry of the domains was 1:2, not 1:1. An electron transfer pathway through the polypeptide chain was proposed, but is perhaps unlikely given the excessive length of the route. Indeed, the search for proteinaceous electron transfer pathways through *P*-450 systems may be a pointless one. A short, through-space route is much more likely in the class II system, with the possible involvement of a small number of amino acids that themselves are hydrogen-bonded or intimately associated with the FMN or heme cofactors, as postulated for the *P*-450cam system [112]. The recently solved crystal structure of rat cytochrome *P*-450 reductase indicates that FAD-to-FMN electron transfer occurs between edges of the flavin ring systems and does not involve a protein pathway [127]. Rotation of the reduced FMN domain of the reductase away from its FAD/NADPH domain is probably necessary in order to expose the FMN for interaction at the heme proximal face and subsequent delivery of electrons to the heme.

In the *P*-450 BM3 heme domain, the cysteine-thiolate ligand to the heme iron (C400) is exposed to solvent at the distal face of the heme iron in the center of a region likely to represent a redox partner docking area. As such, it is accessible for direct electron transfer from FMN through to the heme iron [12,16].

4.5. Alternative reducing sources

All *P*-450 systems rely on NAD(P)H as the ultimate electron donor. However, this is not a cost-effective way to drive *P*-450 reactions, because NADPH is very expensive to produce commercially. There is much interest in developing alternative, cheaper means of driving *P*-450 reactions. These include the use of the 'peroxide shunt' mechanism to deliver oxygen and reducing equivalents simultaneously via an organic peroxide (e.g. cumene hydroperoxide) or hydrogen peroxide. However, such reactions are generally inefficient and result in gradual oxidative destruction of the heme macrocycle by the peroxide. The future of such technology may rest with the design of mutant *P*-450s selected for optimal reactivity with peroxides. The ground breaking study of Joo et al. [128] demonstrated that 'directed evolution' (i.e., successive rounds of random mutagenesis coupled to a selection screen for mutants with desired activity enhancement) could be used to select for mutants of *P*-450cam able to hydroxylate naphthalene. In this study, mutants with hydrogen peroxide-driven activity greater than 20-fold that of wild-type were isolated. This was achieved by co-expression of a peroxidase enzyme that converted the hydroxylated product into easily detectable fluorescent derivatives. This technique is in its infancy, but there are clearly great possibilities for the generation of mutant *P*-450s able to perform industrially or medically important processes that are driven by inexpensive chemicals.

Perhaps an even more exciting prospect is the use of an electrode to drive *P*-450 catalysis. This technique has been pioneered by Faulkner and co-workers in their study of the fatty acid hydroxylase *P*-450 4A1 from rat [129]. The ω-hydroxylation of laurate in a system containing distinct or fused *P*-450 and *P*-450 reductase and the redox mediator cobalt(III) sepulchrate could be driven from an electrode poised at low potential (approx. −650 mV). The system could be run for up to 2 h without significant destruction of *P*-450 activity [130]. The presence of a flavoprotein is essential for transfer of electrons from cobalt sepulchrate, the reduced form of which is regenerated from the electrode. In studies with the progesterone hydroxylase *P*-450c17, Estabrook and coworkers demonstrated that *E. coli* flavodoxin could substitute for *P*-450 reductase in the system, albeit with lower efficiency [131]. These studies demonstrate the potential for the bulk production of chemicals, and again have been greatly simplified by the breakthroughs in heterologous expression that have permitted the high level expression of eukaryotic *P*-450s in *E. coli* (reviewed by Barnes [132]).

4.6. Genetic fusions

P-450 BM3 represents a natural fusion between a reductase and a *P*-450 and is the most catalytically efficient *P*-450 characterized to date. Another area of interest is the construction of 'artificial' fusions be-

tween *P*-450 moieties and their electron transport accessory protein(s). The ultimate goal of such studies would be to reproduce the catalytic efficiency displayed by *P*-450 BM3. Failing that, self-sufficient enzymatic fusions could still be of great biotechnological potential, obviating the need to express and purify independently the component proteins of *P*-450 systems.

4.6.1. Fusions with NADPH-P-450 reductase

The first artificial *P*-450 fusion protein was reported by Murakami et al. in 1987 [133]. This group fused rat *P*-450 1A1 with rat NADPH-*P*-450 reductase to give a functionally active enzyme expressed in yeast. Following on from this success, fusions using the bovine *P*-450s 17A [134] and 21A [135] and yeast NADPH-*P*-450 reductase were generated by the same group. The development of heterologous expression systems for eukaryotic cytochromes *P*-450 in *E. coli* [136] allowed the overexpression of *P*-450 fusion proteins in this bacterium. Fisher et al. [137] reported the fusion of two mammalian *P*-450s, bovine 17A and rat 4A1, with rat liver NADPH-*P*-450 reductase and Shet et al. [138] generated a fusion between the human *P*-450 3A4 and the same reductase. The molecular biology involved in the construction of such fusions has been reviewed elsewhere [139]. The basic premise is that both cDNAs are modified and joined in a single reading frame. The stop codon of the *P*-450 is removed and replaced with a short polypeptide linker encoding a restriction endonuclease site (e.g. Ser/Thr dipeptide linker containing a *Sal*I site [135]). This facilitates the fusing of the *P*-450 with the reductase. The latter is modified at its N-terminus to remove the major membrane binding region (e.g. first 56 residues of rat liver reductase deleted [133]).

In a more recent report, Chun et al. reported the construction of a human *P*-450 1A1/rat reductase fusion expressed in *E. coli* [140]. The study of human *P*-450 1A1 is of interest due to its potential role in activation of carcinogens and its inducibility by some environmental toxins [141,142]. The fused system was constructed essentially as described above, using a Ser/Thr linker (Fig. 8a). *P*-450 reductase from rat was used, since attempts to utilize the human reductase in fusions with human *P*-450s 3A4 and 3A5 had resulted in constructs in which the reductase portion was not fully functional [46,143]. The fusion enzyme was expressed at high levels in *E. coli* and found to be catalytically active either in purified form or within the bacterial cells. Substrate turnover rates were comparable to those for the reconstituted system.

An example of a reported application of a fused rat *P*-450 1A1/yeast reductase protein was described by Shiota et al. [144]. The fusion was expressed in tobacco plants and although its expression level and activity were less than those observed in yeast, it conferred resistance to the herbicide chlortoluron to the plants.

A further demonstration of the usefulness of *P*-450/NADPH-*P*-450 reductase chimeras is seen from studies of a fusion between canine liver *P*-450 2B11 and rat liver NADPH-cytochrome *P*-450 reductase. This was constructed to expedite the analysis of mutated 2B11 enzymes [145]. The 2B11 fusion protein efficiently metabolized androstenedione when assayed as crude sonicated whole cell extracts. Activity and metabolic profiles were comparable with those of purified and reconstituted preparations. In this study, nine mutant forms of 2B11 were generated in the fusion protein and rapidly characterized using sonicated *E. coli* extracts.

4.6.2. Fusions with electron donors other than P-450 reductase

Sibbesen et al. [146] described the first heterologous, self-sufficient catalytic system for oxidation of *P*-450cam substrates. Fusions of the cDNAs of *P*-450cam and its electron donors PdR and Pd (both soluble) were generated and expressed in *E. coli*. The ordering of the components as well as the regions linking them were varied. Activities of the fusion proteins were compared by measuring oxygen consumption in the presence and absence of camphor. The most active of the constructs was that with *P*-450cam at the C-terminus: PdR-Pd-*P*-450cam (in contrast to the *P*-450 reductase fusions) (Fig. 8b), the order of the proteins in the fusion having more of an effect than the nature of the linkers between them. The limiting feature of catalytic turnover was found to be the interaction between *P*-450cam and Pd, most likely due to structural constraints. Evidence for this included the increase in catalytic activity of the fusion upon addition of both exogenous Pd and *P*-450cam and the high activity observed when

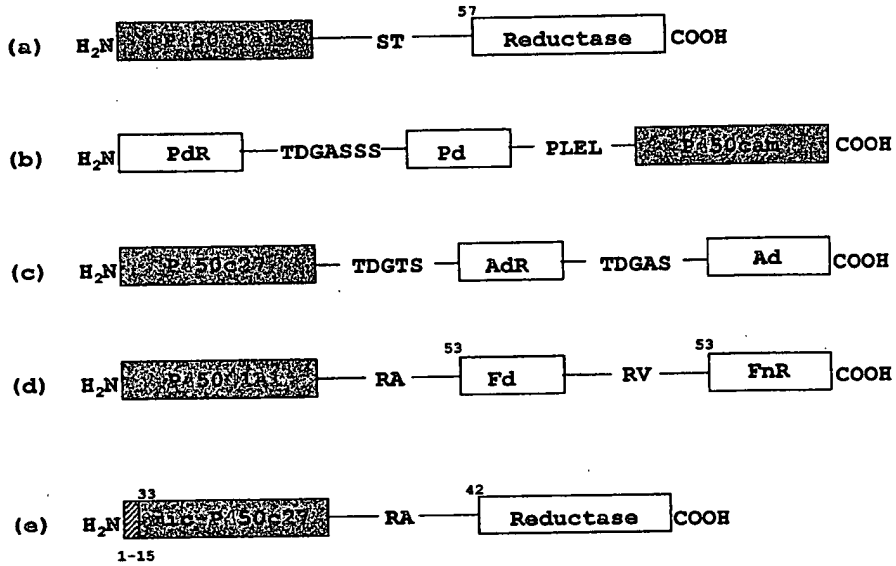


Fig. 8. Schematic representation of the different *P*-450 fusion constructs described showing the variation in linkers used and the ordering of the component proteins. The stop codons between protein pairs are deleted, fusing all components in a single, open reading frame. The amino acids in the linker regions are denoted by their single letter codes. (a) Fusion between human *P*-450 1A1 and (Δ1–56)-rat NADPH-*P*-450 reductase [140]; (b) putidaredoxin reductase (PdR)/putidaredoxin (Pd)/*P*-450cam triple fusion [146]; (c) human *P*-450c27/mature adrenodoxin reductase (AdR)/mature adrenodoxin (Ad) triple fusion [147]; (d) triple fusion between rat microsomal *P*-450 1A1, mature maize ferredoxin I (Fd) and mature pea ferredoxin NADP$^+$ reductase (FnR) [150]; (e) mitochondrial *P*-450c27 N-terminally modified by deleting the mitochondrial targeting sequence (residues 1–32) and replacing it with the microsomal targeting sequence of *P*-450c17 (residues 1–15) (mic-*P*-450c27) fused to (Δ1–41)-yeast NADPH-*P*-450 reductase [151].

P-450cam was incubated with fused PdR/Pd. Because the activity of the triple fusion increases linearly with its concentration the electron transfer from NADPH is considered to occur via an intramolecular pathway. Although this fusion protein is less efficient than the reconstituted wild-type system, the turnover is comparable to that of other reconstituted *P*-450 systems.

Similar fusions, expressed in mammalian COS-1 cells, have also been reported for the class I mitochondrial *P*-450s c27, scc and 11β with their redox partners adrenodoxin and adrenodoxin reductase [147–149]. In the case of *P*-450c27, the fusion (Fig. 8c) was more efficient than the native reconstituted system in transfected mammalian cells.

4.6.3. *Fusions with non-physiological electron donors*

Whereas the above fusions describe *P*-450s in combination with their 'physiological' electron donors, Lacour and Ohkawa [150] have reported a fusion (expressed in yeast) in which rat *P*-450 1A1, a class II *P*-450 normally supplied with reducing equivalents from NADPH-*P*-450 reductase, has been fused to ferredoxin (Fd) and ferredoxin NADP$^+$ reductase (FnR) from plant chloroplasts to recreate an electron transfer chain resembling a class I system. The order of the proteins in the most efficient triple fusion generated was *P*-450-Fd-FnR (Fig. 8d) for all activities assayed. Not surprisingly, activities were lower when compared with *P*-450 1A1 fused to its physiological electron donor NADPH-*P*-450 reductase (e.g. the 1A1/reductase fusion was 5-fold more active with 7-ethoxycoumarin as the substrate than the triple fusion). It was concluded that electron transfer from FnR to Fd was not optimal in the triple fusion due to unfavorable redox potentials. The ultimate aim of generating such a system is to produce mammalian *P*-450s in plant chloroplasts to improve herbicide detoxification (to date, no *P*-450 monooxygenase system has been identified in plant chloroplasts) [150].

Sakaki et al. [151] describe the fusion of a modified class I *P*-450 with the class II microsomal yeast reductase (Fig. 8e). In modified *P*-450c27 (mic-*P*-450c27), the mitochondrial targeting signal was re-

placed by the microsomal targeting sequence of bovine *P*-450c17. This localized the protein to yeast microsomes where it appeared able to function as a microsomal *P*-450 by accepting electrons from NADPH-*P*-450 reductase. To study further electron transfer from NADPH-*P*-450 reductase to mic-*P*-450c27, a fusion between the two proteins was constructed. It was shown that the reductase in the fusion efficiently transferred electrons to the *P*-450, the rate of heme reduction being much faster than observed for the separate enzymes (similar results were obtained from studies with a fusion between rat *P*-450 1A1 and NADPH-*P*-450 reductase [152]). The rate of substrate hydroxylation was also 5-fold greater than that obtained when the two separate proteins were expressed simultaneously.

5. Conclusions

The cytochromes *P*-450 catalyze the monooxygenation of a vast array of substrate types. Some of these reactions have great medical or industrial importance with a huge potential for the application of protein engineering. This review demonstrates the attractive and realistic proposition that protein engineering approaches can be used to modulate cytochromes *P*-450 to catalyze the oxygenation of a molecule of choice. Protein engineering has also been shown to be a key tool for probing the role of active site residues and for dissecting the route of electron transfer through the protein. In addition, the manipulation of properties such as membrane binding and even the possibility of employing alternative reducing sources have been shown to be amenable to protein engineering methods.

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